GEORGIA INSTITUTE OF TECHNOLOGY — OFFICE OF CONTRACT ADMINISTRATION

PROJECT ADMINISTRATION DATA SHEET

[X] ORIGINAL
[ ] REVISION NO. ____

Project No. G33-L04 DATE: 8-7-81

Project Director: James C. Powers School/~~Lab~~ Chemistry

Sponsor: DHEW/PHS/NIH - National Heart, Lung, and Blood Institute

Type Agreement: Grant No. 1-R01-HL 22530-04

Award Period: From 8-1-81 To 7-31-82 (Performance) 10-31-82 (Reports)

Sponsor Amount: $ 57,450 Contracted through:

Cost Sharing: $ 3,373 (G33-367) ~~GTRC~~/GIT

Title: Active Site Studies On Blood Proteases

ADMINISTRATIVE DATA OCA CONTACT Don Hosley

1) Sponsor Technical Contact: Dr James Wyatt, Division of Blood Diseases & Resources, Nat'l Heart, Lung & Blood Institute, Bethesda, Md 20014 - phone (301) 496-5911

2) Sponsor Admin./Contractual Contact: Mrs Gloria Blanton, Grants Operation Branch, Division of Extramural Affairs, Nat'l Heart, Lung, & Blood Institute, Bethesda, Md 20014 phone (301) 496-7255

Reports: See Deliverable Schedule Security Classification: N/A

Defense Priority Rating: N/A

RESTRICTIONS

See Attached NIH Supplemental Information Sheet for Additional Requirements.

Travel: Foreign travel must have prior approval - Contact OCA in each case. Domestic travel requires sponsor approval where total will exceed greater of $500 or 125% of approved proposal budget category.

Equipment: Title vests with N/A - None approved

COMMENTS: Follow on to Project G33-L03.

COPIES TO:

Administrative Coordinator
Research Property Management
Accounting Office
Procurement/EES Supply Services
Research Security Services
~~Reports Coordinator (OCA)~~
Legal Services (OCA)
Library, Technical Reports
EES Research Public Relations
Project File (OCA)
Other: ____

GEORGIA INSTITUTE OF TECHNOLOGY OFFICE OF CONTRACT ADMINISTRATION

SPONSORED PROJECT TERMINATION SHEET

Date 10/19/82

Project Title: Active Site Studies on Blood Proteases

Project No: G-33-L04

Project Director: James C. Powers

Sponsor: DHEW/PHS/NIH - National Heart, Lung and Blood Institute; Bethesda, MD. 20014

Effective Termination Date: 7/31/82

Clearance of Accounting Charges: ----

Grant/Contract Closeout Actions Remaining:

NONE

- ☐ Final Invoice and Closing Documents
- ☐ Final Fiscal Report
- ☐ Final Report of Inventions
- ☐ Govt. Property Inventory & Related Certificate
- ☐ Classified Material Certificate
- ☒ Other Annual Report of Expenditures (04 Year)

NOTE: Continued by G-33-L05

Assigned to: Chemistry (School/~~Laboratory~~)

COPIES TO:

RAN
~~Administrative Coordinator~~
Research Property Management
Accounting
Procurement/EES Supply Services
Research Security Services
~~Reports Coordinator (OCA)~~
Legal Services (OCA)
Library
EES Public Relations (2)
Computer Input
Project File
Other GTRI

G-33-L04

SECTION IV

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1	GRANT NUMBER	
SECTION IV—SUMMARY PROGRESS REPORT	HL 22530-05	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT	
Powers, James C.	FROM	THROUGH
NAME OF ORGANIZATION		
Georgia Institute of Technology	8/1/81	5/27/82

TITLE (Repeat title shown in Item 1 on first page)
Active Site Studies on Blood Proteases

1. List all publications, not previously reported, resulting from work supported by this grant (author(s), title, page numbers, year, journal or book). List manuscripts separately as submitted for publication or accepted for publication.
2. Provide two reprints of publications not previously submitted to the awarding unit.
3. Progress Report. (See instructions)

Final Report G33-L04/Powers

Publications

Mapping the Active Sites of Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, Factor XII_a, Plasma Kallikrein, and Trypsin with Amino Acid and Peptide Thioesters: Development of New Sensitive Substrates, B.J. McRae, K. Kurachi, R.L. Heimark, K. Fujikawa, E.W. Davie, and J.C. Powers, Biochemistry 1981, 20 7196-7206.

Mapping the Substrate Binding Site of Human $C1\bar{r}$ and $C1\bar{s}$ with Peptide Thioesters. Development of New Sensitive Substrates. B. J. McRae, T-Y Lin and J.C. Powers, J. Biol. Chem. 1981, 256 12362-12366.

Progress Report

Scientific Goals. The goal of this research is to understand the nature of the active sites of plasma serine proteases and other related trypsin-like enzymes. These proteins are the basis of important physiological processes such as blood clotting, fibrinolysis and the immune defense mechanism involving the complement system. The tools for the investigation will be synthetic peptides. Synthetic peptides corresponding to sites which are cleaved by various plasma proteases will be prepared. The binding of these peptides to plasma proteases and their rates of hydrolysis will be determined. In addition we plan to design and synthesize small peptide thioesters which are more reactive toward serine proteases than simple peptides. These will be utilized to develop more sensitive and specific assays for individual enzymes.

Progress. During the course of this year we have continued our work with amino acid and dipeptide thioesters. In McRae et al., 1981 (reprint enclosed) we used thioester to study most of the bovine coagulation factors. In the course of this work we developed sensitive substrates for all of the enzymes studied. Of special interest was the development of thioester substrates for factor IX_a, an enzyme for which there was no previous suitable substrate. Several of these thioester substrates are now commercially available. In the last year we have extended these studies to bovine and human protein C, and to human factor XII_a. In addition we have used these same thioester substrates to map the active sites of two other human trypsin-like enzymes, one from human skin and one from human lung. Overall we have now studied over 15 trypsin-like enzymes with the thioester substrates. In terms of reactivity the enzymes can be classified into three groups. The high reactivity group includes enzymes such as skin trypsin, bovine trypsin and kallikrein. The moderate reactivity group includes enzymes such as the lung trypsin, factor X_a and factor XI_a. The low reactivity group includes enzymes such as factor IX_a. The thioester substrates that we have developed are extremely sensitive, but show little specificity.

(continued)

A second avenue of investigation has involved the study of tripeptide 4-nitroanilide substrates. In our thioester work we discovered that the dipeptides which contained either a P_2 Phe or Gly residue were the most reactive. Therefore we decided to synthesize 4-nitroanilide substrates with the following sequences Suc-AA-Gly-Arg-NA and Suc-AA-Phe-Arg-NA. The P_3 amino acid residue (AA) was chosen to represent each of the major classes of amino acids. Seven substrates in each series were synthesized. The amino acids chosen for the P_3 position were Lys, Asn, Ser, Ala, Glu, Pro, and Phe. We have now completed the synthesis of all 14 substrates and are measuring kinetic constants with the various enzymes. Thus far we have studied thrombin, factor XI_a, factor X_a, trypsin, factor IX_a, and factor XII_a. This week the studies with bovine protein C have been completed, although the kinetic results are not yet completely corrected. In the next week or two, we plan to complete the work with human protein C. In addition to the coagulation factors, we have also measured kinetics with the human skin and lung trypsin-like enzymes. The P_3 residue has a substantial effect on the reactivity of the various substrates toward the enzymes which we have investigated thus far. In fact, it was common to observe two or three orders of magnitute difference in k_{cat}/K_M values. The 4-nitroanilide substrates were much less reactive than the thioester substrates and were also more specific. However none of the nitroanilides were completely specific.

The third avenue of investigation has involved the synthesis of peptides containing the activation sequences of bovine factors IX and X and study of their reactions with bovine factors XI_a and IX_a. The substrates contain a fluorophore (Abz) and a quenching group (Nba) which are separated upon enzymatic hydrolysis with a resultant increase in fluorescence which was utilized to measure hydrolysis rates. Factor XI_a cleaved all of the peptides bearing factor IX activation site sequences. The kinetic behavior of factor XI_a toward the synthetic peptide substrates indicates that it has a minimal extended substrate recognition site at least five residues long and has favorable interactions over seven subsites. The hexapeptide Abz-Glu-Phe-Ser-Arg-Val-Val-Nba was the most specific factor XI_a substrate and was not hydrolyzed by factors IX_a, X_a or thrombin. Phospholipids had no effect on the reactivity of either factors IX_a or X toward synthetic substrates. Both factor IX_a and X_a cleaved the peptide substrates at similar rates to their natural substrates under comparable conditions. However, the rates were substantially lower than optimium activation rates observed in the presence of Ca^{++}, phospholipids and protein cofactors. In the future it may be useful to investigate synthetic substrates which can bind phospholipid vesicles in the same manner as the natural substrates for factor IX_a and X_a.

Specific Objectives. The first objective for next year will be to complete the kinetic studies with the thioester and 4-nitroanilide substrates that we have already synthesized. Studies with human protein C and factor XII_a remain to be done. In addition we expect to obtain some acrosin for study later this summer.

Another objective will be to investigate the reaction of thioesters with zymogens. We have synthesized Z-Gly-SBzl and Z-Ala-SBzl, and have discovered that both are hydrolyzed slowly by chymotrypsinogen. We plan to extend these studies to factor X, factor IX, and prothrombin. If these studies are successful, we may try to synthesize longer thioesters to make more reactive substrates and

increase their specificity. These studies could lead to direct assays for coagulation factors without the need of prior proteolytic activation.

Another goal is to study the P' subsites of thrombin. We plan to synthesize Z-Arg-SCH_2CO-Pro-NH_2 and measure kinetics with thrombin. Due to the presence of the P_2' Pro, this substrate may be specific for thrombin and may not react with other coagulation factors. In addition we plan to see if there is any difference between the clotting and non-clotting forms of thrombin.

Our final goal will be to begin to synthesize peptides containing the structural features of Gla residues. Possible structures would include $(HO_2C)_2CHCH_2CH_2CO$-spacer-peptide. Our purpose is to design peptides which could bind to phospholipid vesicles in the same manner as the natural substrates for factor IX_a and X_a. Such substrates might be significantly more reactive and specific than the ones we have prepared to date. One of our major overall goals for the future is to increase the specificity of various substrates.

G-33-LO4

SECTION IV

APPLICANT: REPEAT GRANT NUMBER SHOWN ON PAGE 1	GRANT NUMBER	
SECTION IV—SUMMARY PROGRESS REPORT	HL 22530-05	
PRINCIPAL INVESTIGATOR OR PROGRAM DIRECTOR (Last, First, Initial)	PERIOD COVERED BY THIS REPORT	
Powers, James C.	FROM	THROUGH
NAME OF ORGANIZATION		
Georgia Institute of Technology	8/1/81	5/27/82

TITLE (Repeat title shown in Item 1 on first page)
Active Site Studies on Blood Proteases

1. List all publications, not previously reported, resulting from work supported by this grant (author(s), title, page numbers, year, journal or book). List manuscripts separately as submitted for publication or accepted for publication.
2. Provide two reprints of publications not previously submitted to the awarding unit.
3. Progress Report. (See instructions)

Final Report G33-LO4/Powers

Publications

Mapping the Active Sites of Bovine Thrombin, Factor IX_a, Factor X_a, Factor XI_a, Factor XII_a, Plasma Kallikrein, and Trypsin with Amino Acid and Peptide Thioesters: Development of New Sensitive Substrates, B.J. McRae, K. Kurachi, R.L. Heimark, K. Fujikawa, E.W. Davie, and J.C. Powers, Biochemistry 1981, 20 7196-7206.

Mapping the Substrate Binding Site of Human $C1\bar{r}$ and $C1\bar{s}$ with Peptide Thioesters. Development of New Sensitive Substrates. B. J. McRae, T-Y Lin and J.C. Powers, J. Biol. Chem. 1981, 256 12362-12366.

Progress Report

Scientific Goals. The goal of this research is to understand the nature of the active sites of plasma serine proteases and other related trypsin-like enzymes. These proteins are the basis of important physiological processes such as blood clotting, fibrinolysis and the immune defense mechanism involving the complement system. The tools for the investigation will be synthetic peptides. Synthetic peptides corresponding to sites which are cleaved by various plasma proteases will be prepared. The binding of these peptides to plasma proteases and their rates of hydrolysis will be determined. In addition we plan to design and synthesize small peptide thioesters which are more reactive toward serine proteases than simple peptides. These will be utilized to develop more sensitive and specific assays for individual enzymes.

Progress. During the course of this year we have continued our work with amino acid and dipeptide thioesters. In McRae et al., 1981 (reprint enclosed) we used thioester to study most of the bovine coagulation factors. In the course of this work we developed sensitive substrates for all of the enzymes studied. Of special interest was the development of thioester substrates for factor IX_a, an enzyme for which there was no previous suitable substrate. Several of these thioester substrates are now commercially available. In the last year we have extended these studies to bovine and human protein C, and to human factor XII_a. In addition we have used these same thioester substrates to map the active sites of two other human trypsin-like enzymes, one from human skin and one from human lung. Overall we have now studied over 15 trypsin-like enzymes with the thioester substrates. In terms of reactivity the enzymes can be classified into three groups. The high reactivity group includes enzymes such as skin trypsin, bovine trypsin and kallikrein. The moderate reactivity group includes enzymes such as the lung trypsin, factor X_a and factor XI_a. The low reactivity group includes enzymes such as factor IX_a. The thioester substrates that we have developed are extremely sensitive, but show little specificity.

(continued)

A second avenue of investigation has involved the study of tripeptide 4-nitroanilide substrates. In our thioester work we discovered that the dipeptides which contained either a P_2 Phe or Gly residue were the most reactive. Therefore we decided to synthesize 4-nitroanilide substrates with the following sequences Suc-AA-Gly-Arg-NA and Suc-AA-Phe-Arg-NA. The P_3 amino acid residue (AA) was chosen to represent each of the major classes of amino acids. Seven substrates in each series were synthesized. The amino acids chosen for the P_3 position were Lys, Asn, Ser, Ala, Glu, Pro, and Phe. We have now completed the synthesis of all 14 substrates and are measuring kinetic constants with the various enzymes. Thus far we have studied thrombin, factor XI_a, factor X_a, trypsin, factor IX_a, and factor XII_a. This week the studies with bovine protein C have been completed, although the kinetic results are not yet completely corrected. In the next week or two, we plan to complete the work with human protein C. In addition to the coagulation factors, we have also measured kinetics with the human skin and lung trypsin-like enzymes. The P_3 residue has a substantial effect on the reactivity of the various substrates toward the enzymes which we have investigated thus far. In fact, it was common to observe two or three orders of magnitute difference in k_{cat}/K_M values. The 4-nitroanilide substrates were much less reactive than the thioester substrates and were also more specific. However none of the nitroanilides were completely specific.

The third avenue of investigation has involved the synthesis of peptides containing the activation sequences of bovine factors IX and X and study of their reactions with bovine factors XI_a and IX_a. The substrates contain a fluorophore (Abz) and a quenching group (Nba) which are separated upon enzymatic hydrolysis with a resultant increase in fluorescence which was utilized to measure hydrolysis rates. Factor XI_a cleaved all of the peptides bearing factor IX activation site sequences. The kinetic behavior of factor XI_a toward the synthetic peptide substrates indicates that it has a minimal extended substrate recognition site at least five residues long and has favorable interactions over seven subsites. The hexapeptide Abz-Glu-Phe-Ser-Arg-Val-Val-Nba was the most specific factor XI_a substrate and was not hydrolyzed by factors IX_a, X_a or thrombin. Phospholipids had no effect on the reactivity of either factors IX_a or X toward synthetic substrates. Both factor IX_a and X_a cleaved the peptide substrates at similar rates to their natural substrates under comparable conditions. However, the rates were substantially lower than optimium activation rates observed in the presence of Ca^{++}, phospholipids and protein cofactors. In the future it may be useful to investigate synthetic substrates which can bind phospholipid vesicles in the same manner as the natural substrates for factor IX_a and X_a.

Specific Objectives. The first objective for next year will be to complete the kinetic studies with the thioester and 4-nitroanilide substrates that we have already synthesized. Studies with human protein C and factor XII_a remain to be done. In addition we expect to obtain some acrosin for study later this summer.

Another objective will be to investigate the reaction of thioesters with zymogens. We have synthesized Z-Gly-SBzl and Z-Ala-SBzl, and have discovered that both are hydrolyzed slowly by chymotrypsinogen. We plan to extend these studies to factor X, factor IX, and prothrombin. If these studies are successful, we may try to synthesize longer thioesters to make more reactive substrates and

increase their specificity. These studies could lead to direct assays for coagulation factors without the need of prior proteolytic activation.

Another goal is to study the P' subsites of thrombin. We plan to synthesize Z-Arg-SCH_2CO-Pro-NH_2 and measure kinetics with thrombin. Due to the presence of the P_2' Pro, this substrate may be specific for thrombin and may not react with other coagulation factors. In addition we plan to see if there is any difference between the clotting and non-clotting forms of thrombin.

Our final goal will be to begin to synthesize peptides containing the structural features of Gla residues. Possible structures would include $(HO_2C)_2CHCH_2CH_2CO$-spacer-peptide. Our purpose is to design peptides which could bind to phospholipid vesicles in the same manner as the natural substrates for factor IX_a and X_a. Such substrates might be significantly more reactive and specific than the ones we have prepared to date. One of our major overall goals for the future is to increase the specificity of various substrates.